

COMMUNICATIONS OF THE DEPARTMENT OF ANATOMY
UNIVERSITY OF LUND, SWEDEN
No 2. 1973

HISTOCHEMISTRY OF FLUOROGENIC AMINES AND PEPTIDES
WITH NH₂-TERMINAL TRYPTOPHAN IN POLYPEPTIDE
HORMONE-SECRETING CELLS

With special reference to the calcitonin cells

BY

FRANK SUNDLER

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AKADEMISK AVHANDLING

som med vederbörligt tillstånd av medicinska fakulteten i
Lund för avläggande av medicine doktorexamen kommer
att offentligen försvaras å Histologiska avdelningens före-
läsningssal (nya byggnaden), Institutionen för anatomi och
histologi, Lund, tisdagen den 17 april 1973 kl. 9.15 (prick)

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FRANK SUNDLER

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From the Institute of Anatomy and Histology
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Lund 1973

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HISTOCHEMISTRY OF FLUOROCITRIC AMIDES AND PEPTIDES
WITH 1- α -TERMINAL TRYPTOPHAN IN POLYESTER
HORMONE-SECRETING CELLS

With special reference to the endocrine cells

FRANK JONSSON



The present summary is based on the following papers:

Part I

- I Larson, B., Ch. Owman and F. Sundler: Monoaminergic mechanisms in parafollicular cells of the mouse thyroid gland.
Endocrinology 78: 1109-1114 (1966).
- II Owman, Ch. and F. Sundler: Indole metabolism in thyroid C cells of the mouse: effects of thyrocalcitonin and thiouracil.
In: *Calcitonin. Proceedings of the Symposium on Thyrocalcitonin and the C cells*, Taylor, S. (Ed.), p. 110-121, London: Heinemann (1968).
- III Melander, A., Ch. Owman and F. Sundler: Effect of vitamin D₂ stimulation on amine stores and secretory granules in the calcitonin cells of the mouse.
Histochemie 25: 32-44 (1971).
- IV Almqvist, S., E. Malmqvist, Ch. Owman, M. Ritzén, F. Sundler and G. Swedin: Dopamine synthesis and storage, calcium-lowering activity, and thyroidal properties of chicken ultimobranchial cells.
Gen. Comp. Endocrinol. 17: 512-525 (1971).
- V Melander, A., Ch. Owman and F. Sundler: Concomitant depletion of dopamine and secretory granules from cells in the ultimobranchial gland of vitamin D₂-treated chicken.
Histochemie 25: 21-31 (1971).
- VI Englund, N.E., G. Nilsson, Ch. Owman and F. Sundler: Human thyroid C cells: occurrence and amine formation studied by perfusion of surgically removed goitrous glands.
J. Clin. Endocr. Metab. 35: 90-96 (1972).
- VII Håkanson, R., Ch. Owman and F. Sundler: Aromatic L-amino acid decarboxylase in calcitonin-producing cells.
Biochem. Pharmacol. 20: 2187-2190 (1971).

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- VIII Håkanson, R., Ch. Owman, B. Spörrong and F. Sundler: Electron microscopic classification of amine-producing endocrine cells by selective staining of ultra-thin sections.
Histochemie 27: 226-242 (1971).

Part II

- IX Håkanson, R. and F. Sundler: Fluorometric determination of N-terminal tryptophan-peptides after formaldehyde condensation.
Biochem. Pharmacol. 20: 3223-3225 (1971).
- X Edvinsson, L., R. Håkanson and F. Sundler: Separation of tryptophyl-peptides by thin-layer chromatography and microspectrofluorometric characterization of their formaldehyde-induced fluorophores.
Anal. Biochem. 46: 473-481 (1972).
- XI Håkanson, R. and F. Sundler: Formaldehyde condensation. A method for the fluorescence microscopic demonstration of peptides with NH_2 -terminal tryptophan residues.
J. Histochem. Cytochem. 19: 477-482 (1971).
- XII Håkanson, R. and F. Sundler: Formaldehyde-induced fluorescence of a tryptophyl tetrapeptide.
J. Histochem. Cytochem. 19: 693-695 (1971).
- XIII Håkanson, R., A.-K. Sjöberg and F. Sundler: Formaldehyde-induced fluorescence of peptides with N-terminal 3,4-dihydroxyphenylalanine or 5-hydroxytryptophan.
Histochemie 28: 367-371 (1971).
- XIV Håkanson, R., Ch. Owman and F. Sundler: Fluorescence histochemical and microspectrofluorometric evidence of tryptophyl peptides in thyroid C cells of cat and pig.
J. Histochem. Cytochem. 20: 205-210 (1972).

- XV Håkanson, R., K. Lindstrand, Ch. Owman and F. Sundler: Tryptamine or tryptophyl-peptides in endocrine cells of rabbit gastric antrum. *Biochem. Pharmacol.* 21: 1703-1712 (1972).
- XVI Håkanson, R., L.-I. Larsson, A. Nobin and F. Sundler: Tryptamine or tryptophyl peptides in endocrine cells of the mammalian adenohypophysis? *J. Histochem. Cytochem.* 20: 908-916 (1972).
- XVII Håkanson, R., A. Melander, Ch. Owman and F. Sundler: Depletion of secretory granules, calcitonin, and formaldehyde-ozone-induced fluorescence from cat thyroid C cells by vitamin D₂ treatment. *Histochemie*. In press.

These papers will be referred to by their Roman numerals.

INTRODUCTION

In 1876 Baber described a cell system — the parenchymatous cells — in the mammalian thyroid gland, which could be distinguished from the follicle cells by their size and shape and by their finely granulated cytoplasm. That the thyroid gland contained a second system of epithelial cells was confirmed by Nonidez (1932 a and b), who showed that what he called the "parafollicular cells" could be distinguished from the follicle cells by their strong argyrophilia, i.e. they could be stained with silver in the presence of an extraneous reducing agent. Soon after the discovery of calcitonin, a blood calcium-lowering polypeptide hormone that can be isolated from extracts of the mammalian thyroid (cf. Copp 1969, Hirsch and Munson 1970), reports appeared suggesting that it emanated from the parafollicular cells. This was proven by Bussolati and Pearse (1967) who showed by immunofluorescence that calcitonin in pig and dog occurred in these cells, which were therefore referred to as C cells.

In lower vertebrates (i.e. birds, reptiles, amphibians and fish), the calcitonin-producing cells constitute a separate organ, the ultimobranchial gland, situated caudally to the thyroid, close to the parathyroids and the carotid body (cf. Copp 1969, Bélanger 1971).

In their studies on pancreatic islet cells, Falck and Hellman (1963) were first to show that cells known to produce polypeptide hormones also store monoamines (in the islet cells of guinea-pig later identified as 5-hydroxytryptamine (5-HT) and dopamine; Cegrell, Falck and Rosengren 1967), histochemically demonstrable by their formaldehyde-induced fluorescence. These findings gave a stimulus to further studies concerning the occurrence of amines in polypeptide hormone-secreting cells in general (see Owman, Håkanson and Sundler 1973). Moreover, it was early demonstrated that the immediate amine precursors DOPA and 5-HTP were taken up into various polypeptide hormone-producing endocrine cells (Hempel 1963, Ritzén, Hammarström and Ullberg 1965, Gershon and Ross 1966, Pearse 1966). It was also observed that in many such cells the exogenous amine precursors were converted to the corresponding amines, which were stored intracellularly. This occurred also in polypeptide hormone-producing cells

which were devoid of endogenous fluorogenic amines (Papers I and II; Cegrell 1968, Falck and Owman 1968).

Endocrine cells producing polypeptide hormones have several ultrastructural features in common, such as a well-developed Golgi apparatus, narrow arrays of endoplasmic reticulum often concentrated to one part of the cell, numerous free ribosomes and microtubules and microfilaments. One conspicuous characteristic is the presence of numerous membrane-bound cytoplasmic granules (Pearse 1968, 1969, Fawcett, Long and Jones 1969).

This treatise is based on a series of investigations on amines and on peptides with NH_2 -terminal tryptophan in certain polypeptide hormone-secreting cells. Since it had been shown that parafollicular cells in the sheep thyroid store high concentrations of 5-HT (Falck, Larson, Mecklenburg, Rosengren and Svenaeus 1964), the thyroid gland of a variety of species was examined for the presence of fluorogenic amines. An unexpected finding was that parafollicular cells of most laboratory animals – including mice – were devoid of histochemically visible fluorogenic amines. A common feature was the ability to synthesize and store dopamine or 5-HT upon administration of DOPA or 5-HTP respectively (Papers I and II). At this time, it was shown that the parafollicular cells were responsible for the formation of the polypeptide hormone calcitonin (Bussolati and Pearse 1967). Our interest was therefore focused on the fate of the amine during activation of the calcitonin cells (C cells) both in an animal – mouse – in which these cells normally are devoid of fluorogenic amines (Paper III) and in an animal – chicken – where C cells contain dopamine and constitute a separate organ, the ultimobranchial gland which is easily accessible for study (Papers IV and V).

As human C cell tumors – medullary carcinoma – contain fluorogenic amines (Falck, Ljungberg and Rosengren 1968), studies were also performed on amine mechanisms in C cells of the normal human thyroid gland (Paper VI). The activity and distribution of the amine-forming enzyme (aromatic L-amino acid decarboxylase) were examined chemically in thyroid and ultimobranchial tissue from various species (Paper VII). In order to allow comparison of the subcellular characteristics

of C cells with other amine-producing endocrine cells, techniques were developed for localization of catecholamines and 5-HT by performing certain staining reactions directly on ultra-thin sections (Paper VIII).

The studies indicated that C cells of all species were capable of forming and storing amines, though a fluorogenic amine (a catecholamine or 5-HT) could be demonstrated only in a few species. Since modifications of the formaldehyde technique had offered possibilities to visualize also tryptamine and 3-methoxylated phenylethylamines (Björklund, Falck and Håkanson 1968, Björklund and Stenevi 1970), a search for such compounds in C cells was made. Application of these modifications showed that combined formaldehyde-ozone treatment, appropriate for visualization of tryptamine, induced strong whitish-yellow fluorescence in the feline and porcine C cells (which are devoid of catecholamines and 5-HT). Accordingly, the fluorescence was initially thought to reflect tryptamine, but this amine could not be demonstrated chemically; a more detailed analysis indicated that the fluorogenic compound was instead a peptide having tryptophan in NH_2 -terminal position. The conditions under which such peptides form fluorophores with formaldehyde, and their spectral properties, were therefore investigated in a series of chemical and histochemical model experiments (Paper IX-XII). An additional observation was that not only peptides with tryptophan in NH_2 -terminal position, but also peptides with DOPA or 5-HTP in NH_2 -terminal position formed fluorophores with formaldehyde (Paper XIII). Tissue application of results obtained in model experiments indicated that peptides with NH_2 -terminal tryptophan occurred in three different endocrine cell systems – C-cells of cat and pig (Paper XIV), argyrophil cells (probably gastrin-producing) in rabbit pyloric antrum (Paper XV), and in certain cells of the adenohypophysis (Paper XVI). The large amounts of tryptophyl peptides in the hypophysis allowed a more detailed chemical analysis of the fluorogenic compound.

Finally, studies were carried out to elucidate signs of any physiological significance of the tryptophyl-peptide by studying the effect of vitamin D_2 stimulation on the intensity of formaldehyde-ozone-induced fluorescence and the calcitonin content of the cat C cells (Paper XVII).

RESULTS AND COMMENTS

1. Formation, storage and release of amines

1.1 Amines in thyroid C cells of the mouse

The C cells of the mouse thyroid did not contain any histochemically detectable fluorogenic amine. However, after injection of L-DOPA, which is the immediate precursor of the fluorogenic amine dopamine, an intense green formaldehyde-induced fluorescence accumulated in the C cell cytoplasm (Paper I). The identity of the cellular fluorophore was not immediately evident, since not only dopamine, but also DOPA itself, emit identical fluorescence (Jonsson 1967). Following inhibition of the decarboxylating enzyme however, little or no fluorescence appeared in the C cells after exogenous L-DOPA. This indicated that the amino acid did not accumulate in the C cells and that the fluorescence in the first-mentioned experiment was due to the decarboxylated product. This was later confirmed chemically (Paper III). Since no accumulation of dopamine could be observed if the amine was administered, the results indicated that the decarboxylation of DOPA occurred within the C cell.

Pretreatment of animals with reserpine, known to block amine-storing mechanisms in adrenergic nerves, did not prevent the appearance of an intense fluorescence in the C cells after DOPA. However, the distribution of the fluorescence was different. It was not restricted to the cytoplasm but occurred also over the nucleus, indicating that the amine normally accumulates in a reserpine-sensitive storage mechanism. Another observation pointing in this direction was that, following reserpine treatment the fluorescence rapidly disappeared from the cells.

Pretreatment of animals with a monoamine oxidase (MAO) inhibitor markedly increased the fluorescence intensity induced by administration of L-DOPA. Moreover, the fluorescence remained in the C cells for a much longer period after MAO inhibition, the intensity being high for at least 20 hours after the L-DOPA injection. The presence of MAO in the C cells could be established

histochemically with the tetrazolium method.

A series of related experiments with 5-HTP as amine precursor (Paper II), gave results which were very similar to those obtained with DOPA, indicating that the mouse thyroid C cells can synthesize 5-HT upon administration of the immediate precursor amino acid 5-HTP, and that the amine formed is stored in the cytoplasm by a reserpine-sensitive mechanism in the same way as dopamine. Attempts were made in preliminary experiments to find evidence of a relation between the amine and the hormone by studying the effect of exogenous calcitonin on the accumulation of 5-HT in the C cells after administration of 5-HTP. When animals were given calcitonin before 5-HTP, the fluorescence intensity in the C cells was markedly lower than in controls given 5-HTP alone. If the hormone was given afterwards, the fluorescence intensity was higher than in the controls. The results suggest that reduction of the C cell activity through high levels of exogenous calcitonin may affect the turnover rate of amine and exogenous amine precursor in the C cells.

This might explain the finding of a prolonged retention of 5-HT in the C cells of mice pretreated with propylthiouracil, since hypothyroidism induced in this way impairs the release of calcitonin (Duncan and Care 1967).

In a search for further evidence of a functional relation between the amine and the polypeptide hormone, the formation of dopamine from exogenous DOPA was studied during vitamin D-induced activation of the C cells by a combination of fluorescence histochemistry, electron microscopy and chemical determinations of dopamine (Paper III). In agreement with previous ultrastructural investigations by others (Wissig 1962, Azzali 1964, Ekholm and Ericson 1968, Nanba and Fujita 1969) the C cells were found to contain numerous electron-dense cytoplasmic granules. Treatment of mice with vitamin D for 3-5 days caused a pronounced degranulation of the C cells together with signs of cellular activation, *i.e.* enlargement of the Golgi apparatus, proliferation of the endoplasmic reticulum, and increased number of free ribosomes. Concomitantly, the content of dopamine (induced by L-DOPA administration) in the C cells was markedly reduced compared with control animals receiving no vitamin D. The vitamin D-induced change

in the content of dopamine was specific in that no such alteration was observed in other amine-storing endocrine cells, such as the enterochromaffin-like cells in the oxyntic gland area of the stomach (Håkanson, Lilja and Owman 1967). After inhibition of MAO, however, the dopamine content could be restored in the degranulated C cells. This finding was interpreted as indicating the presence of another amine-storage mechanism, apart from the electron-dense granules. When the vitamin D treatment was combined with reserpine, the C cells still contained numerous secretory granules with an appearance similar to control animals or to animals given reserpine alone. It is possible that the reserpine-sensitive amine storage mechanism is obligatory for the vitamin D-induced degranulation.

1.2 Dopamine in chicken C cells

The C cells in the chicken differ from those in mice in that they form a discrete organ, the ultimobranchial gland, and that they contain an endogenous fluorogenic amine, dopamine, which gives the cells an intense formaldehyde-induced green fluorescence (Paper IV). The content of dopamine showed marked variation from cell to cell. After injection of L-DOPA the over-all fluorescence intensity was increased; all cells showing the same intense fluorescence. A rapid formation of dopamine in the ultimobranchial tissue was observed in incubation experiments using tritiated DOPA; autoradiography suggested that the amine was synthesized locally within the cell. Treatment of chicken with reserpine caused a depletion of the dopamine content in the C cells. This is in accordance with findings on mice suggesting that the amine-storing mechanism in the C cells of these two species is similar.

The possible effect of increased rate of hormone secretion on the dopamine content was studied after vitamin D-induced stimulation of the C cells (Paper V). The techniques used were essentially the same as those reported in similar studies on the mouse (Paper III), although those previous studies concerned dopamine formed from exogenous DOPA.

The vitamin D treatment induced a marked depletion of dopamine from the C cells, simultaneous with a disappearance of the cytoplasmic granules. Administration of DOPA to vitamin D-treated animals resulted in a reappearance of dopamine in the cytoplasm of the degranulated C cells, in analogy with the findings on the mouse C cells (Paper III). This was taken as an indication of the presence of a cytoplasmic amine storage mechanism other than the cytoplasmic granules. Similar observations were subsequently made in the rat C cells (Dahlström and Ericson 1972). The vitamin D treatment did not reduce the 5-HT content in cells of the carotid body, included in the preparations, indicating that the hypercalcemia (Ericson 1968) selectively affected the C cells.

1.3 Amine formation in human thyroid C cells

The human medullary carcinoma is a tumor arising from C cells. The tumor cells show formaldehyde-induced fluorescence, and 5-HT has been identified chemically in extracts of the tumor (Falck, Ljungberg and Rosengren 1968). Unexpectedly, however, no fluorogenic amine was found in the normal human C cells. It was of interest therefore to study the possible formation and storage of amines from exogenous precursors in non-malignant human thyroid tissue, which could be obtained after surgical removal (Paper VI). To maintain the tissue in vital condition, thyroid lobes were perfused with oxygenated blood. In specimens taken from thyroid lobes perfused with blood containing L-DOPA, an intense green formaldehyde-induced fluorescence was observed in scattered or clustered cells, identified as C cells by their argyrophilia upon silver staining. Chemical determinations showed the presence of dopamine in DOPA-perfused (but not in non-perfused) glands. Since no other fluorescent structures appeared after the perfusion, it is probable that the newly formed dopamine was present in the C cells and responsible for the green formaldehyde-induced fluorescence. A notable finding was that the number of C cells was significantly higher in toxic than in non-toxic goiters.

1.4 Aromatic L-amino acid decarboxylase activity in calcitonin-producing cells

In order to obtain a more direct information about the formation of amines within the thyroid, the activity of aromatic L-amino acid decarboxylase (DOPA decarboxylase) in the thyroid gland of mouse, rat, rabbit, guinea-pig, cat, pig, sheep, and chicken, and in the chicken ultimobranchial gland was compared with the number of C cells in the glands (Paper VII). The enzyme catalyses the conversion of DOPA and 5-HTP to the corresponding amines dopamine and 5-HT, and was measured radiometrically using ^{14}C -labelled DOPA as substrate (cf. Håkanson 1970).

The thyroid DOPA decarboxylase activity was found to vary markedly from one species to another. From cell counting it could be established that the enzyme activity and the number of C cells in general showed a remarkable agreement. The chicken thyroid, which is devoid of C cells, had no enzyme activity, suggesting that the follicle cells do not contain DOPA decarboxylase. The chicken ultimobranchial gland and the sheep thyroid, in which the C cells contain an endogenous fluorogenic amine, had conspicuously high DOPA decarboxylase activity compared with the thyroids of the other species studied. The results from cell countings indicate that the enzyme activity per cell is higher in the former than in the latter species.

The enzyme activity was also measured within different regions of the mouse thyroid and was found to agree with the number of C cells in these regions. The results support the view that thyroid DOPA decarboxylase is present in the C cells and that the thyroid follicle cells have no or very low activity of the enzyme. It seems unlikely therefore, that DOPA decarboxylase plays any major role in the metabolism of the follicle cell as has previously been suggested (Schulz and Oliner 1967).

1.5 Ultrastructural identification of polypeptide hormone-secreting cells

Several staining reactions, particularly modifications of chromium and silver staining techniques, have been used to identify and classify amine-producing endocrine cells in the light microscope. The chromaffin and argentaffin reactions are known to reveal the presence of reducing substances, such as catecholamines and 5-HT, when they occur in high concentrations in the cell. The argyrophil reaction on the other hand, which involves the use of an extraneous reducing agent, probably reflects a high affinity for silver to granular components of unidentified chemical nature. It can be assumed that this high affinity for silver is a prerequisite also for a positive argentaffin reaction.

When applied at the electron microscopic level, these staining reactions have usually been performed en bloc, and therefore do not allow comparison between stained and unstained sections – or between differently stained sections – from the same specimen. In attempts to make such comparisons possible, staining reactions were performed directly on ultrathin sections (Paper VIII). With this technique it was found that the 5-HT-storing (enterochromaffin) cells in the gastric mucosa and the catecholamine-storing adrenal medullary cells were argyrophil and also strongly argentaffin and chromaffin. The electron-dense precipitate formed was almost exclusively located over the cytoplasmic granules in the cells.

The argentaffin and chromaffin reactions could be induced with sections from glutaraldehyde- as well as formaldehyde-fixed material; post-fixation with osmium tetroxide prevented the staining reactions. The argyrophil reaction, on the other hand, could be performed successfully only on sections from formaldehyde-fixed specimens, in agreement with findings at the light microscopic level. The C cells of the mouse thyroid have previously been shown to be strongly argyrophil at the light microscopic level (Paper III). This was now confirmed at the electron microscopic level and, as in the enterochromaffin cells and the adrenal medullary cells, the precipitate was located over the cytoplasmic granules. As could be expected, the argentaffin and chromaffin reactions were negative. Pretreatment

of the animals with DOPA or 5-HTP induced argentaffinity and chromaffinity of the secretory granules in the mouse C cells. The staining intensity showed marked variation between the C cells in one and the same section and also between individual granules in one and the same cell, probably reflecting differences in the amine content.

The findings on the mouse C cells support the view that positive argentaffin and chromaffin reactions reflect the presence of reducing amines and suggest that also amines formed from exogenous precursors are accumulated and stored together with calcitonin in the secretory granules.

II Fluorescence histochemistry of peptides with NH_2 -terminal tryptophan

II.1 Model experiments with peptides having tryptophan in NH_2 -terminal position

Fluorometric determinations. The fluorescence characteristics of tryptamine, tryptophan and some tryptophan-containing dipeptides condensed with formaldehyde in aqueous solution according to Hess and Udenfriend (1959) were compared (Paper IX). Their method was originally introduced for the fluorometric determination of tryptamine, and involves the use of hydrogen peroxide as oxidant.

All tested peptides having NH_2 -terminal tryptophan were found to give strong fluorescence after combined treatment with formaldehyde and hydrogen peroxide; no fluorescence developed if the formaldehyde condensation or the oxidation step was omitted. Peptides having tryptophan in COOH -terminal position did not give fluorescence with formaldehyde-hydrogen peroxide treatment. The peptide fluorophores showed spectral properties very similar to those of tryptamine and tryptophan. The fluorescence intensity was proportional to the concentration of the peptide within a wide range, and was generally somewhat lower than for equimolar concentrations of tryptamine and tryptophan.

Silica gel thin-layer models. In a preliminary study (Håkanson and Sundler 1971), aqueous solutions of some tryptophan-containing dipeptides, as well as tryptamine and tryptophan, were spotted onto silica gel thin-layers, treated with

gaseous formaldehyde at 100°C for 30–60 min and examined in UV light. Tryptamine and all peptides with tryptophan in NH₂-terminal position were found to emit strong whitish–yellow fluorescence, while the colour of the tryptophan fluorophore was blue. None of the dipeptides with tryptophan in COOH-terminal position gave fluorescence upon formaldehyde treatment. These preliminary findings were confirmed and somewhat extended in a series of model experiments (Paper X), in which attempts were made to establish reaction conditions for the formation of fluorophores and their spectral characteristics after condensation with formaldehyde. Tryptophan, as well as several dipeptides and one tetrapeptide, all having NH₂-terminal tryptophan, were used as test substances. The following detection reagents were used: aqueous formaldehyde, gaseous formaldehyde, Procházka I (acidified formaldehyde solution) Procházka II (ammoniacal formaldehyde solution) and Procházka II + I (Procházka II treatment followed by Procházka I treatment). All the reagents tested, except Procházka II, were found to give strong fluorescence with tryptophan as well as with the peptides, permitting detection of submicrogram amounts of the substances. The Procházka II treatment on the other hand was conspicuously less sensitive. The fluorescence intensity showed only small variations between the different dipeptides. It is notable that, on an equimolar basis, the intensity of the tetrapeptide fluorescence was in the same range as for the dipeptides. The emission maximum of the tryptophan fluorophore differed markedly from those of the peptides except after treatment with the Procházka I reagent. With this latter treatment, the fluorophores of tryptophan and the peptides showed similar spectral characteristics with a marked concentration-dependent shift in the emission maximum, from 450 mμ at low concentrations to 550 mμ at high concentrations.

Protein droplet models. In a search for possibilities to differentiate between tryptamine and tryptophyl-peptides, different reaction conditions for tryptamine were applied to these substances enclosed in protein droplets. In these experiments, albumin solutions containing various tryptophan-containing dipeptides, tryptamine or tryptophan were sprayed as microdroplets onto ordinary glass cover slips (Paper XI). The dried protein droplet models were subjected to three different formalde-

hyde condensation reactions: standard Falck-Hillarp procedure (formaldehyde gas 1 hr 80°C), combined formaldehyde-ozone treatment (graded amounts of ozone together with formaldehyde in the reaction vessel; Björklund, Falck and Håkanson 1968), and combined formaldehyde HCl treatment (graded amounts of HCl vapour together with formaldehyde in the reaction vessel; Björklund and Stenevi 1970). Treatment with formaldehyde alone gave only a weak blue fluorescence, whereas combined formaldehyde-ozone treatment resulted in intense yellow fluorescence from all the peptides having NH_2 -terminal tryptophan. The peptides with tryptophan in COOH-terminal position yielded no visible fluorescence. The maximal fluorescence intensity was obtained with an amount of ozone in the reaction vessel corresponding to 20-40 min of electric discharge. The tryptophyl-peptides thus behaved much like tryptamine upon this treatment, with a marked increase in fluorescence intensity compared to after treatment with formaldehyde alone. Furthermore, the spectral properties of the peptide and tryptamine fluorophores showed great similarities, with emission maximum at 440-480 m μ after treatment with formaldehyde alone and 500-520 m μ after combined formaldehyde-ozone treatment. Tryptophan, on the other hand, behaved differently from the peptides and also from tryptamine in that the fluorescence yield did not increase significantly upon combined formaldehyde-ozone treatment.

Combined formaldehyde-HCl treatment produced intense fluorescence with tryptamine, while the intensity of the tryptophyl peptide fluorescence was less markedly increased, compared with exposure to formaldehyde alone. The intensity of the tryptophan fluorescence after formaldehyde-HCl treatment was intermediate between that of tryptamine and the tryptophyl peptides. For all substances tested, the optimal concentration of HCl in the reaction vessel corresponded to a partial pressure of 100-300 torr.

The spectral characteristics of the fluorophores of the different tryptophyl-peptides were very similar and resembled those of the tryptamine fluorophore. The emission maxima showed similar concentration dependent red-shift as has previously been shown for the tryptamine fluorophore (Björklund, Falck and Håkanson

1968).

The formaldehyde-induced fluorescence of a larger peptide, the gastrin COOH-terminal tetrapeptide (tetragastrin), was similarly studied in aqueous solution, on silica gel thin-layers and in protein droplet models (Paper XII). The findings obtained with this peptide resembled the previous observations on tryptophyl-dipeptides, thus indicating that combined formaldehyde-ozone treatment gives optimal conditions for the histochemical demonstration also of larger peptides with NH_2 -terminal tryptophan.

11.2 Model experiments with peptides having other fluorogenic amino acids in NH_2 -terminal position

The finding that peptides having NH_2 -terminal tryptophan gave formaldehyde-induced fluorescence with spectral characteristics very similar to that of tryptamine suggested that also peptides with NH_2 -terminal DOPA or 5-HTP might form fluorophores with formaldehyde and that their spectral properties would resemble those of the corresponding amine (dopamine and 5-HT, respectively). A few dipeptides having COOH- or NH_2 -terminal DOPA or 5-HTP were synthesized and the development of formaldehyde-induced fluorescence was tested on silica gel thin-layers (DOPA- and 5-HTP-peptides) and in protein droplet models (DOPA-peptides) (Paper XIII). Peptides with NH_2 -terminal DOPA or 5-HTP were found to emit strong fluorescence upon standard formaldehyde treatment (see also Rorsman, Rosengren and Rosengren 1971), whereas peptides having COOH-terminal DOPA or 5-HTP gave no formaldehyde-induced fluorescence. The spectral properties of the DOPA-peptide fluorophores were very similar to those of the dopamine (and DOPA) fluorophores, and the spectra from the 5-HTP-peptide fluorophores agreed with those of the 5-HT (and 5-HTP) fluorophores. The fluorescence intensity exhibited by the different peptides were comparable with equimolar concentrations of dopamine and 5-HT, respectively.

11.3 Evidence for the occurrence of peptides with NH_2 -terminal tryptophan in endocrine cells

Thyroid C cells of the cat and pig. Combined formaldehyde-ozone treatment induced strong greenish-yellow fluorescence in an extensive system of epithelial cells in the thyroid of cat and pig, but not in other species examined (Paper XIV). The cells were argyrophilic and thus classified as C cells. In order to identify the fluorogenic substance, the yield and spectral properties of the cellular fluorescence was compared with that of tryptamine and tryptophyl-glycine enclosed in protein microdroplets after standard formaldehyde treatment, combined formaldehyde-ozone treatment or combined formaldehyde-HCl treatment. Under these reaction conditions, the spectral properties of the cellular fluorescence closely resembled those of the tryptamine and tryptophyl-peptide fluorophores. Standard formaldehyde treatment gave weak fluorescence in the C cells as well as in the tryptamine and the tryptophyl-glycine models, whereas combined formaldehyde-ozone treatment yielded strong fluorescence in the C cells and in the models. Combined formaldehyde-HCl treatment on the other hand induced a strong fluorescence with tryptamine, while C cell fluorescence and the fluorescence of tryptophyl-glycine were only moderately improved. No tryptamine-like material could be detected chemically in extracts from cat and pig thyroid glands.

Taken together, these data suggest that the fluorogenic substance stored in the C cells of cat and pig thyroid is a peptide (or peptides) with NH_2 -terminal tryptophan rather than tryptamine.

Endocrine cells of the rabbit gastric antrum. The pyloric gland area of the rabbit stomach has been found to contain a population of argyrophil cells of presumed endocrine nature, storing a substance that exhibit weak formaldehyde-induced fluorescence, which differs from that of catecholamines and 5-HT (Håkanson, Owman, Sjöberg and Spörng 1970). The fluorescence intensity in these cells was found to be markedly improved after combined formaldehyde-ozone treatment (Paper XV). The possibility that also this fluorescence, like that in the cat and pig C cells,

reflected the presence of a tryptophyl peptide was examined in a series of experiments in which the yield of cellular fluorescence was compared with that of tryptamine and of tryptophyl-glycine after treatment with formaldehyde alone, formaldehyde-ozone or formaldehyde-HCl. The results obtained were very similar to those described above for the cat thyroid C cells. Thus, the spectral characteristics of the cellular fluorescence showed close resemblance to those of the tryptamine and tryptophyl-glycine fluorophores in protein droplet models. Combined formaldehyde-ozone treatment markedly intensified the cellular fluorescence as well as that of tryptamine and tryptophyl-glycine, while combined formaldehyde-HCl gave intense fluorescence only with tryptamine. The fluorescence histochemical and microspectrofluorometric results suggested the presence of a peptide (or peptides) with NH_2 -terminal tryptophan in the endocrine-like cells of the antro-pyloric mucosa. This assumption was supported by the failure to detect tryptamine-like material chemically in extracts of this tissue.

Endocrine cells in mammalian adenohypophysis. The first observations of a formaldehyde-induced fluorescence in the mammalian adenohypophysis were made by Pearse and McGregor (1964) and Dahlström and Fuxe (1966). Because of the weakness of the fluorescence the exact colour was difficult to establish. For this reason the substance responsible for the fluorescence was by the former investigators believed to be 5-HT, whereas the latter suggested that it was a primary catecholamine. Subsequently, Björklund and Falck (1969 a and b) showed that the fluorescence yield in these cells was markedly improved if the formaldehyde condensation took place in the presence of ozone, or if the sections were acidified after standard formaldehyde treatment. With these treatments the fluorescence turned bright yellow. The conditions under which the fluorescence developed, and its spectral properties, suggested that the cellular fluorescence reflected the presence of a tryptamine-like compound rather than a catecholamine or 5-HT. However, tryptamine was not determined chemically in pituitary extracts. The observation that peptides with NH_2 -terminal tryptophan yielded formaldehyde-induced fluorescence with properties similar to those of the tryptamine fluorophore seemed to offer an alter-

native explanation, namely that the formaldehyde-induced fluorescence in the adenohypophysis reflected the presence of a peptide with NH_2 -terminal tryptophan. This was tested histochemically by comparing the yield and spectral properties of cellular fluorescence with that of tryptamine and tryptophyl-alanine after formaldehyde condensation under different reaction conditions (Paper XVI). The results obtained confirmed those made by Björklund and Falck (1969 a and b) and showed that the histochemical properties of the cellular fluorophore in all respects agreed with those of tryptophyl peptides in protein droplets. Attempts to demonstrate tryptamine chemically failed. That the cellular fluorescence reflected a tryptophyl-peptide rather than tryptamine was corroborated in fluorometric determinations showing the presence of large amounts of tryptophyl peptides (30-40 $\mu\text{g/g}$ of tryptophyl-alanine equivalents) in extracts of pig pituitary. The fluorogenic peptides were purified by gel chromatography and shown to have high molecular weight. The spectral properties of their formaldehyde-induced fluorophores closely resembled those of some known tryptophyl peptides as analyzed on silica gel thin-layers.

Depletion of secretory granules, calcitonin and formaldehyde-ozone-induced fluorescence from cat C cells. In order to get some idea of the nature of the fluorogenic substance in the cat thyroid C cells, the influence of vitamin D treatment was studied by fluorescence histochemistry and electron microscopy in combination with fluorometric determinations of tryptophyl-peptides and bioassay of calcitonin (Paper XVII).

In agreement with previous observations in other species, vitamin D treatment caused a marked degranulation of the C cells concomitant with a depletion of the calcitonin content. At the same time the formaldehyde-ozone-induced fluorescence disappeared. The amount of tryptophyl-peptides were determined fluorometrically in the granular fractions obtained by differential centrifugation of thyroid homogenates. The amount of tryptophyl-peptides in the granule fraction from the control animals was larger than in the corresponding fraction from vitamin D-treated animals. These findings support the assumption that the fluorogenic substance stored in the cat thyroid C cells is a peptide (or peptides) with NH_2 -terminal tryptophan, that it is stored in the cytoplasmic granules in these cells, and that

it is released together with calcitonin as a result of C cell stimulation.

DISCUSSION

The development some ten years ago of a procedure for the fluorescence histochemical demonstration of monoamines opened up unique possibilities for the exact cellular localization of this group of biologically important substances (cf. Björklund, Falck and Owman 1972). The identification of fluorogenic amines at the cellular level has been greatly facilitated by the introduction of microspectrographic techniques (Ritzén 1967, Björklund, Ehinger and Falck 1968, Björklund, Falck and Stenevi 1971), permitting the registration of excitation and emission spectra of intracellular fluorophores.

By these methods it was possible to demonstrate that a variety of polypeptide hormone-secreting cells contained large amounts of arylethylamines such as dopamine or 5-HT (cf. Falck and Owman 1968, Håkanson, Lundquist, Melander, Owman, Sjöberg and Sundler 1972, Owman, Håkanson and Sundler 1973). In many such cells, however, no fluorogenic amines could be detected. Nevertheless, it now seems well established, that also those polypeptide hormone-secreting cells devoid of endogenous fluorogenic amines have the capacity to produce and store such amines, if supplied with the corresponding precursor amino acid (Cegrell 1968, Falck and Owman 1968, Pearse 1968, Håkanson 1970, Lundquist 1971, Tjälve 1971, Ericson 1972, Ericson, Håkanson, Larson, Owman and Sundler 1972). The functional implications of the difference between these latter cells and cells storing endogenous fluorogenic amines are unknown.

Endocrine cells producing polypeptide hormones have several ultrastructural features in common. The most conspicuous one is the presence in their cytoplasm of numerous secretory granules. The shape, size and electron density of the granules usually show characteristic differences between different endocrine cells. Cell fractionation studies and immunohistochemistry at the electron microscopic level have given strong support to the view that the granules represent the storage

site of the hormone (Bauer and Teitelbaum 1966, Cooper and Tashjian 1966, Misugi, Howell, Greider and Sorenson 1970, Nakane 1970, De Grandi, Kraehenbuhl and Campiche 1971, Kalina and Pearse 1971, Atack, Ericson and Melander 1972, Greider, Steinberg and McGuigan 1972, Moriarty and Halmi 1972).

The proper identification of amine-storing endocrine cells in the electron microscope has been facilitated by applying staining procedures, originally developed for light microscopic studies, such as the argyrophil and argentaffin reactions, directly to ultrathin sections thus permitting the different stainings to be performed on consecutive sections from one and the same specimen.

Moreover, the development of techniques for the localization of aryl-ethylamines at the ultrastructural level such as autoradiography (Ericson 1970, Tjälve 1971, Nunez and Gershon 1972) and histochemical procedures that make use of the reducing capacity of these substances (Jaim-Etcheverry and Zieher 1968, Chang and Bencosme 1968) have allowed a more detailed analysis of the localization of the amine within the cell. On the basis of findings with such techniques, together with chemical determinations on cell fractions, it seems now well documented that the amine is stored in the cytoplasmic granules which contain the peptide hormone (cf. Atack, Ericson and Melander 1972).

A problem that remains to be solved is the significance of the seemingly random variability in most endocrine cell systems with respect to the type of amine stored. Thus, in the horse (Solcia and Sampietro 1968), sheep (Falck, Larson, Mecklenburg, Rosengren and Svenaeus 1964), goat (Falck and Owman 1968), bat (Gershon and Nunez 1970) and duck (Sundler, unpublished) the C cells store 5-HT, whereas in cow (Melander, Sundler and Westgren 1973) and chicken they store DA. In the C cells of several other species, including man, no amine at all can be demonstrated. The formation and storage of histochemically demonstrable fluorogenic amines (after administration of the corresponding precursor amino acid) in the C cells also of these latter species have been suggested to "mimic" the handling of an endogenous, but hitherto unidentified, amine in these cells (cf. Falck and Owman 1968, Owman, Håkanson and Sundler 1973).

The role of the intracellular amine in endocrine cell function is a matter of considerable interest and controversy. It has been proposed that the amine is somehow involved in the storage and/or release of the polypeptide hormone (Pearse 1966, Kalina and Pearse 1969, Ullberg and Hammarström 1969, Lernmark 1971, Lundquist 1971, Tjälve 1971, Ericson 1972). For example, high levels of 5-HT or dopamine within the β -cells have been found to inhibit stimulated insulin release probably by an action within the cells (Lernmark 1971, Lundquist 1971). Further, pretreatment of mice with reserpine causes marked impairment of the insulin secretion in vitro in response to various stimuli (Lernmark 1971), a finding that may be analogous to the inhibitory action of reserpine on the C cell degranulation upon treatment with vitamin D (Paper III). In addition, a direct correlation seems to exist between the concentration of amine and hormone in the insulin-producing cells of guinea-pig under certain experimental conditions (Cegrell, Falck and Hellman 1964, Håkanson, Lundquist, Melander, Owman, Sjöberg and Sundler, 1972, Håkanson, Lundquist and Sundler 1973). Such findings, together with the observations of concomitant changes in the amine level and the number of secretory granules in the C cells after vitamin D stimulation favour the view that the amine is functionally involved in the process of hormone secretion.

There was reason to suspect that the intense formaldehyde-ozone-induced fluorescence observed in the thyroid C cells of cat and pig, in argyrophil cells of the rabbit antro-pyloric mucosa and in cells of the adenohypophysis reflected the presence of tryptamine, primarily because the spectral properties of the cellular fluorescence showed close resemblance with those of tryptamine. However, certain observations made this assumption less plausible. Firstly, no tryptamine could be detected chemically in extracts of any of the tissues containing cells with formaldehyde-ozone-induced fluorescence. Secondly, the fluorescence showed no tendency to diffuse when the formaldehyde condensation was performed under conditions of high humidity, in contrast to the amine fluorescence which is easily diffusible. Thirdly, reserpine treatment of cats caused no reduction in the amount of fluorogenic substance in the C cells (Paper XIV) or in the adenohypophysis (Björklund and Falck 1969 b) as opposed to the usually marked reduction of the amine content in such

cells that follows treatment with reserpine. The present investigation has shown that, besides the biogenic monoamines, also peptides with certain arylethylamino acids – such as tryptophan, DOPA or 5-HTP – in NH_2 -terminal position can condense with formaldehyde to yield intensely fluorescent products. It is most probable that the chemical reaction mechanisms involved in the formaldehyde condensation of peptides is identical with that previously suggested for the corresponding amino acids and amines (for references see Jonsson 1971). The amino acid composition of the remainder of the peptide seems to have no major influence on the fluorescence yield and spectral properties of the fluorophores formed. The results of microspectrofluorometric analysis have thus led us to suggest that the cellular formaldehyde-ozone-induced fluorescence reflect the presence of peptides with NH_2 -terminal tryptophan. From the hypophysis it has been possible to extract large amounts of peptides with NH_2 -terminal tryptophan, the amounts being large enough to allow further chemical analysis. Corroborating histochemical observations (Björklund, Håkanson, Lindvall and Sundler 1973) have been made with a recently developed method (Björklund, Lindvall and Svensson 1972, Axelsson, Björklund, Falck, Lindvall and Svensson 1973) in which formaldehyde is replaced by glyoxylic acid in the condensation reaction. Thus, glyoxylic acid was found to give intense fluorescence in protein droplet models with peptides exhibiting fluorescence with standard formaldehyde treatment (peptides with NH_2 -terminal DOPA or 5-HTP), or with combined formaldehyde-ozone treatment (peptides with NH_2 -terminal tryptophan). In addition, the glyoxylic acid-induced fluorescence of tryptophyl-peptides shows spectral properties very similar to those obtained from the cat and pig thyroid C cells as well as from the endocrine cells in the rabbit antrum and the adenohypophysis.

Analogous to the formaldehyde-induced fluorescence of certain peptides, o-phthalaldehyde (OPT) has been found to induce strong fluorescence not only with histamine but also with peptides having NH_2 -terminal histidine such as glucagon and secretin (Brody, Håkanson, Owman and Sundler 1972, Edvinsson, Håkanson, Rönnberg and Sundler 1972, Håkanson, Owman and Sundler 1972 b).

These findings offered a rational explanation for the OPT-induced fluorescence in the pancreatic α_2 cells (Takaya 1970, Brody, Håkanson, Lundquist, Owman and Sundler 1973). The spectral properties of this fluorescence show close similarities to that of authentic glucagon in models. It is also noteworthy, that the OPT-induced fluorescence in the glucagon cells shows no tendency to diffuse, while this is a significant problem with histamine (Brody, Håkanson, Owman and Sundler 1972). In contrast to the substance giving OPT-induced fluorescence in the pancreatic α_2 cells (most probably the hormone itself), the biological significance of the proposed peptides exhibiting formaldehyde-ozone-induced fluorescence is still obscure. Results obtained with the cat C cells suggest that the fluorogenic substance in these cells is stored in the cytoplasmic granules, and that it is released together with calcitonin upon activation of the cell.

CONCLUSION

The formation of amines and their accumulation in cytoplasmic granules together with the stored secretory product is characteristic of many endocrine cells elaborating polypeptide hormones, for example the calcitonin cells. There is some evidence, though at present only scattered, that the intracellular amine plays a cytophysiological role, conceivably linked with the formation, storage, and/or release of the hormone.

It has been demonstrated that formaldehyde condensation allows histochemical detection not only of catecholamines and 5-HT (and their immediate precursor amino acids) but also of peptides having a fluorogenic amino acid in NH_2 -terminal position. Such peptides (with NH_2 -terminal tryptophan) appear to be present in large amounts in certain polypeptide hormone-producing cells, and in the calcitonin cells it has been shown that this peptide disappears together with the hormone during vitamin D-induced activation of the cells.

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ACKNOWLEDGEMENTS

This work has been supported by grants from the Medical Faculty, University of Lund, from the Swedish Medical Research Council (Projects No. 04X-3547 and 04X-3764) and from Albert Pahlsson's Foundation, and has been carried out within a research organization sponsored by the Swedish Medical Research Council (Project No. 04X-712).

